PROCESS FOR PARTITIONING OF PROTEINS

Field of the invention

5   The present invention relates to isolation and purification of proteins in aqueous two-phase systems (ATPS). Specifically the invention provides processes for partitioning of molecules of interest in ATPS by fusing said molecules to targeting proteins which have the ability of carrying said molecule into one of the phases.

10   **Background of the invention**

Liquid-liquid extraction in an aqueous two-phase system (ATPS) can offer a powerful technique for isolation and purification of proteins. The separation of macromolecules and particles by means of liquid-liquid extraction is well known (Albertsson, 1986; Walter *et al.*,

15   1985; Kula, 1990). Mainly polyethylene glycol (PEG) - salt, PEG-dextran and PEG-starch systems have been in use. More recently detergents and detergents with reversed solubility were discovered as suitable methods for separation of macromolecules, and especially for the separation of proteins.

20   An advantage of aqueous two-phase systems (ATPS) is that they are especially suited for large scale processing of microbial proteins not only from culture supernatants but also from crude extracts containing cells and cell debris (Kula, 1979; Kula, 1985). Characteristic features of biological fluids as well as suspensions are rather small particle sizes, low density differences between fluid and suspended solids, high viscosities of the extracts and high

25   compressibility of the solids (Hustedt *et al.*, 1985; Bender and Koglin, 1986). These attributes decrease the performance of conventional methods for solid-liquid separation like centrifugation and filtration at the beginning of a protein recovery process. Using an aqueous two-phase system removal of solids can be integrated into a liquid-liquid separation step, clarification is thus combined with an initial purification (Kula, 1979; Kula, 1985).

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After the extraction process phase separation can be accomplished by settling under gravity as well as by centrifugation (Kula, 1985). ATPS can be applied in various scales from very

small laboratory scale to large industrial scale thus suiting for various proteins, purposes and needs. With regard to industrial purposes commercially available centrifugal separators can be used to shorten separation time. Several authors have investigated the potential of centrifugal separators of various design for processing of large volumes of aqueous two 5   phase systems (Kula, 1979; Kula *et al.*, 1981, Kula *et al.*, 1982; Kula, 1985). In these studies the authors have used polymer/polymer or polymer/salt systems and the results of these investigations demonstrate the feasibility of continuous separation of aqueous two-phase systems in centrifugal separators.

10   Extraction systems based on nonionic surfactants have been described as an alternative to standard polymer/polymer or polymer/salt systems. Phase forming surfactants are e.g. polyoxyethylene type nonionic detergents. The basis of this type of aqueous two-phase system is the temperature-dependent reversible hydration of the polar ethylene oxide head groups. The temperature at which the phase separation occurs is referred to as the cloud 15   point (cloud-point extraction). This kind of aqueous two-phase system is especially suited for the extraction of amphiphilic biomolecules. The potential of this type of two-phase system for separating membrane bound proteins from cytosolic and peripheral membrane proteins was first demonstrated by Bordier (1981). Heusch and Kopp (1988) have been able to demonstrate that lamellar structures formed in the miscibility gaps of polyglycol ether / 20   water systems are responsible for the selective extraction of hydrophobic substances.

Recently, the successful application of a surfactant-based aqueous two-phase system for the extraction of a membrane bound protein (cholesterol oxidase) from the unclarified culture medium of the gram-positive microorganism *Nocardia rhodochrous* on a bench scale has 25   been reported (Minuth *et al.*, 1995). By addition of only one chemical compound a product release through solubilization was possible in homogeneous phase and in a second step a clarification as well as an initial purification was achieved by an extraction process at elevated temperatures separating the detergent rich phase. A closed concept was further developed for the production of the membrane bound enzyme by surfactant-based 30   extraction, organic solvent extraction and anion-exchange chromatography, which gave a product suitable for analytical applications (Minuth *et al.*, 1996).

In aqueous two phase systems the desired target e.g. a protein should partition selectively into one phase (preferentially the lighter phase) while the other substances should partition into the other phase (preferentially the heavier phase). In PEG/salt and PEG/dextran and similar systems there are several driving forces for a substance like charges, hydrophobic, hydrophilic forces or the dependence on conformation or ligand interaction (Albertsson, 1986). The forces leading to separation in detergent based aqueous two phase systems are suggested to be primarily hydrophobic (Terstappen *et al.*, 1993). Even if a lot of work has been carried out in the field of prediction in ATPS, none of the designed models provides a physical picture of the phase behaviour and prediction is hardly possible (Johansson *et al.*, 1998).

In ATPS the partitioning coefficient is defined as the concentration (activity in case of an enzyme) of the target in the top phase divided by the concentration (enzyme:activity) of the target protein in the bottom phase. Partitioning coefficients in ATPS systems are usually in the range from less than 1 up to less than 100 (Terstappen *et al.*, 1992; Terstappen *et al.*, 1993).

$$K = \frac{c_{i,T}}{c_{i,B}}$$

Yield: is defined as the amount of target in the top phase divided by the sum of the amount of target in top and bottom. This leads to the following equation

$$Y_T = \frac{1}{1 + \left[\dfrac{V_B}{V_T} \cdot \dfrac{1}{K}\right]}$$

If the desired substance is directed to the heavier phase (as it can be the case using Triton) the yield is defined by

$$Y_B = \frac{1}{1 + \left[\dfrac{V_T}{V_B} \cdot K\right]}$$

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The volume ratio of the two coexisting phases are defined by the volumes of the lighter over the heavier phase, respectively.

$$R = \frac{V_T}{V_B}$$

5   An example of useful proteins facing problems in purification in a cost-effective way are the commonly used industrial enzymes used as biocatalysts, the glycosyl hydrolases, proteases and lipases produced by fungi and bacteria. These are used in e.g. laundry, textile, paper and pulp, food and feed industry. The fact that microbes produce many different enzymes during their growth and the fact that some of these may be undesired in certain applications leads

10   to a need to enrich the active component(s). This enrichment can be performed by choosing appropriate growth conditions, by genetic engineering and/or by down-stream processing (e.g. purification of the active component(s)).

Purification of proteins are generally performed by chromatography. Usually gel-

15   chromatographic methods are used based on ion-exchange, hydrophobic interaction, affinity chromatography and molecular sieving. Methods like electrophoresis and crystallisation can also be used. These methods are well known in the art and suitable for proteins of fairly high market value. In case of bulky enzyme production these methods, however, are too expensive in order to keep the final product on a compatible price level. Due to similar

20   properties of these enzymes several purification steps are usually needed to separate the proteins from each other. This often causes low final yields and therefore a high loss of product.

Many extracellular hydrolases produced by the filamentous fungus *Trichoderma* are

25   currently used in different industrial applications in large scale. These hydrolases are e.g. hemicellulases (such as xylanases and mannanases), cellulases (such as endoglucanases and cellobiohydrolases) and proteases. Purification of these is well known in the art (Bhikhabhai *et al.*, 1984; Pere *et al.*, 1995), but for large industrial applications the purification methods are too expensive. Alternative methods to enrich these hydrolases have been used, including

30   deletion of undesired genes by genetic engineering (Suominen *et al.*, 1992). However, even

after extensive genetic engineering some minor undesired activities may still be present in the final product.

ATPS have been studied in purification of cellulases of *T. reesei* and the purification of an endoglucanase III showed some promising results, enriching the yield of the protein in the upper phase (U.S. Pat. 5,139,943). ATPS have also been studied in purification of lipases, endoxylanase and natamycin (EP 0 574 050 A1). No K and Y values are, however, mentioned.

As in other protein purification methods, similar properties of proteins produced by an organism impair also in ATPS, e.g. selective separation of one protein is not achieved optimally. To obtain selectivity in purification affinity chomatographic methods are used especially for analytic purposes and in purification of high-value products. These include immunoaffinity chromatography and various fusion protein strategies well described in the art such as fusing the protein of interest to an other protein (e.g. glutathione-S-transferase), protein domain (e.g. protein A-ZZ domain) or small peptide (e.g. His-tag), which selectively bind to the solid carrier and thus the recovery of the fusion partner is obtained as well. The fusion protein can be suitable for the particular purpose as such or cleavage of the product from the added fusion partner may be desired. There are well-known methods in the art on cleavage of fusion proteins from their partners by proteases, e.g. by factor X, thrombin or papain or by genetically introducing a protease cleavage site (e.g. Kex2 site) or autoprosessing domains (e.g. Intein, New England Biolabs) or by chemical cleavage (e.g. CNBr).

ATPS offer advantages mainly with respect to technology compared with the solid state based separation systems e.g. affinity column-based techniques. The scale-up of extractive enzymes is relatively simple utilising commercially available equipment and machinery common in the chemical industry. In addition, it can be used in a continuous process and it can be relatively cost extensive. It can be used as a single step for clarification, concentration and purification. ATPS can be used as a first capture-step, but for bulk products often no further purification is needed.

To aid selective separation in two-phase systems, recent publications have described the fusion of small peptide tags of 12 amino acids to the protein to be purified. The most successful of these soluble peptides are containing tryptophans. So far they have mainly been applied for very small molecules like the staphylococcal protein A derivative ZZT0 (Berggren *et al.*, 1999; Hassinen *et al.*, 1994; Köhler *et al.*, 1991).

Use of ATPS has so far been limited to certain targets. Due to the advantages of ATPS in protein separation, purification and localisation, highly selective and powerful methods should be developed. This is especially important for large scale processes where ATPS in general is very inexpensive as a first capture step or as the only step for purification, clarification and concentration. The system should be universal so that the technique would be strong enough to mediate separation of in principle any component to the desired phase irrespective of its size or biochemical properties.

Description of the invention

In this invention we describe selective separation and partitioning of molecules and particles fusing them with targeting proteins having the capability to carry the molecule or particle of interest to the desired phase in ATPS, and to keep it in this phase if wanted. This invention is directed to make ATPS usable for every biotechnological product. By addition of the targeting protein to selective products, either by genetic tagging of proteins, by chemical binding, glueing or by use of any other technique, the product molecule can be turned more suitable for separation in ATPS. Using ATPS the product or certain component is therefore driven to one phase while the other components or by-products are directed to the other phase(s).

We also describe that efficient separation in ATPS can be obtained using targeting proteins which are/can be larger than the described small soluble synthetic peptide tags of 12 amino acids or less. These targeting molecules can aid in separating of small molecules but even large proteins and particles. Unlike the small peptide tags, it is not necessary that they contain tryptophan residues, although they may do so. They can be hydrophobic or moderately hydrophobic and/or amphipathic in nature, either in monomeric form or when

forming aggregates. Such proteins can be found in nature or they can be designed, or obtained through for instance methods known in art for mutant generation, gene shuffling or directed evolution. Suitable targeting molecules can be screened for instance by fusion the product of interest to a library of natural or mutant sequences, and screening the ability

5   of the fusion molecules to separate in ATPS. Furthermore, any molecule capable of separating in ATPS is a suitable targeting molecule.

Several techniques exploiting purified protein for isolation of the corresponding gene may be used to find genes encoding suitable targeting molecules for ATPS. Suitable proteins or

10   polypeptides may be purified on the basis of their properties. They can be obtained by applying the cells, cell extracts or culture media to ATPS and recovering the proteins or peptides separated into the phase containing the hydrophobic phase material. Suitable targeting molecules may also be recovered for example from the culture medium foam formed either during the cultivation of a microorganism or caused by bubbling gas through

15   the medium. Proteins and peptides suitable as targeting molecules may further be recovered from aggregates caused by freezing of culture media. After the targeting molecules have been purified, the corresponding genes are isolated using techniques known to a person skilled in the art. Such techniques include for example screening of expression libraries using antibodies raised against purified polypeptide or peptide, and PCR cloning and screening of

20   genomic and/or cDNA libraries with oligonucleotides designed on the basis of N-terminal or internal protein sequences.

Examples of molecules suited as targeting proteins in ATPS found in nature are hydrophobin-like small proteins. Hydrophobins are secreted proteins with interesting

25   physico-chemical properties that have recently been discoverd from filamentous fungi (Wessels, 1994; Wösten and Wessels, 1997; Kershaw and Talbot, 1998). They are usually small proteins, approximately 70 to 160 amino acids, containing eight cysteine residues in conserved pattern, and do usually not contain tryptophanes. However, also multimodular proteins with one or several hydrophobin domains and e.g. proline-rich or asparagine/glycine

30   repeats, or hydrophobins containing less than eight cysteine residues have been characterized (Lora et al., 1994; Lora et al., 1995; Arntz and Tudzynski, 1997). Hydrophobins have been divided into two classes based on their hydropathy profiles (Wessels, 1994).

Today most protein data exists for the hydrophobins Sc3p of *Schizophyllum commune* (class I), and cerato-ulmin of *Ophiostoma ulmi* and cryparin of *Cryponectria parasitica* (class II), although more than 30 gene sequences for hydrophobins have been published (Wösten and

5   Wessels, 1997). HFB genes are often naturally highly expressed but due to special requirements in cultivation conditions and the biochemical properties of the proteins, purification of HFBs in large amounts have been difficult. For instance only relatively moderate production levels of a few mg per liter of Sc3 hydrophobin in static cultures are obtained (Han Wösten, personal communication). Published purification procedures include

10 e.g. multi-step extraction from fungal cell walls using organic solvents and bubbling or freezing of culture filtrates (Wessels, 1994). No reports of successful production of hydrophobins are available; levels of cerato-ulmin were no higher than those obtained with other naturally occuring fungal isolates (Temple *et al.*, 1997).

15 Upon shaking hydrophobin-containing solutions, the protein monomers form rodlet-like aggregates. These structures are similar to the ones found on surfaces of aerial stuctures. The self-assembly of purified Sc3 hydrophobin into a 10 nm thick amphipatic layer on hydrophilic and hydrophobic surfaces has been demonstrated (Wösten *et al.*, 1994a; Wösten *et al.*, 1994b). This film is very strongly attached to the surface and not broken, for instance,

20 by hot detergent. The hydrophobic side of the layer on hydrophilic surfaces shows properties similar to teflon (Wessels, 1994). The Sc3 assemblages, as well as those of cerato-ulmin and cryparin, also form on gas-liquid or gas-air interphases thus stabilizing air bubbles or oil droplets in water.

25 Surface activity of proteins is generally low but hydrophobins belong to surface-active molecules, their surfactant capacity being at least similar to traditional biosurfactants such as glycolipids, lipopetides/lipoproteins, phospholipids, neutral lipids and fatty acids (Wösten and Wessels, 1997). In fact Sc3 hydrophobin is the most potent biosurfactant known. It lowers the water surface tension to 24 mJm^2 at a concentration of 50 μg/ml due to a

30 conformational change during self-assembly of monomers into an amphipathic film (Wösten and Wessels, 1997).

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Hydrophobin-like molecules vary in their properties. For instance, rodlet-forming capacity has not been assigned for all hydrophobins (such as some class II), or they might have a weaker tendency to form stable aggregates (Russo et al., 1992; Carpenter et al., 1992). Another group of fungal amphiphatic proteins are repellents (Wösten et al., 1996 (Ustilago),

5   for review, see Kershaw and Talbot, 1998). Consequently, other type of proteins suited as targeting proteins for ATPS, may have only some of the features assigned to hydrophobins. Other suitable proteins are hydrophobic ones such as e.g. lipases, cholesterol oxidase, membrane proteins, small peptide drugs like nisin, aggregating cell wall proteins, lipopetides or any parts of these or combinations of these, and other molecules like glycolipids,

10   phospholipids, neutral lipids, fatty acids in combination with proteins or peptides.

In this invention the targeting protein, such as a hydrophobin-like protein or parts of it, is bound to the product molecule or the component to be separated. First, phase forming materials and eventually possibly also additional salts are added to a watery solution

15   containing the fusion molecule or component, and optionally also the contaminating materials. The added agents are mixed to facilitate their solubilization. As soon as they are solubilized the two phases are formed either by gravity settling or centrifugation. In the separation the target protein drives the product to for instance the detergent-rich phase which could either be the top or the bottom phase. The method is not only useful for

20   purification of products of interest but also for keeping the product or the component of interest, such as a biocatalyst, in a particular phase which enables certain useful biotechnical reactions.

Several ATPS systems are suitable for performing this invention. These include PEG

25   containing systems, detergent based systems and novel thermoseparating polymers. Detergent based systems can be nonionic, zwitterionic, anionic or kationic. The system can be based on amphiphilic polymeric detergents, micelle forming polymers. Novel polymers can be based on polyethylene-polypropylene copolymers such as pluronic block copolymers, Brij, polyoxyethylene derivatives of partial ethers of fatty acids made by adding polyoxyet-

30   hylene chains to the nonesterified body and polyoxyethylene derivatives. The well known PEG/salt, PEG/dextran and PEG/starch (or derivatives such as Reppal, hydroxipropyl starch) systems where PEG and water are forming the top phase and dextran/starch/salt and

water are forming the bottom phase. As salts are used phosphate, citrate, sulfate or others. In the present process the target is partitioning mainly to the top phase, while most of the contaminants are separating mainly to the bottom phase. Some hydrophobic contaminants might partition to the top phase as well. Using detergent based systems only one phase

5   forming detergent has to be added. Optionally, salts and other chemicals can be used in addition. The mentioned chemicals are added, and the solution is mixed. After mixing the separation can take place either by centrifugation or gravity settling. In order to separate into two phases the temperature of the solution has to be over the cloud-point of the detergent. The solution has to be heated if the cloud-point is not reached otherwise. If

10   wanted, a second separation step can follow after a first extraction step and the product rich phase can be further purified. Also the remaining product in the product poor and by-product rich phase can be re-extracted. Very good K values can be obtained and the yields and concentration factors are high.

15   The process of the present invention can advantageously be used in laboratory scale but is especially suitable for large scale separations. It can successfully be used in the separation of proteins and components from large fermentations. Using genetic modifications, the method can be used to purify any protein of interest including extracellular enzymes and proteins such as cellulases and hemicellulases from mixtures containing large amounts of

20   protein such as several grams per liter. Furthermore, this separation can be obtained from various culture media including industrial media containing particular materials such as cellulose and spent grain. The method can be used to purify the product from culture media of strains modified not to produce endogenous hydrophobins. The separation can be done directly from the fermentation broth which can additionally contain cells, even viscous

25   filamentous fungi. High biomass levels are acceptable for the process as explained in example 9. An example is the extracellular endoglucanase I from the fungus *Trichoderma reesei* which can be tagged for instance with the class 2 HFBI and can for example be separated with the nonionic polyoxyethylene C12-C18EO5. In this example the detergent rich phase is the lighter phase and contains most of the tagged endoglucanase while most

30   of the other cellulases, proteases and other enzymes remain in the heavier phase. The mycelium separates to the bottom phase, too. The separation can be achieved using

separation temperatures higher than 25°C. The temperature can be decreased if certain salts like NaCl or K_2SO_4 are added.

5   The invention describes separation of molecules produced in various different organisms such as bacteria, yeast and filamentous fungi. The invention is suitable for purification of product molecules from extra- or intracellular locations, including cell wall bound molecules. It provides examples how the fusion molecule can be secreted by these different organisms but also provide an example how the fusion can be produced intracellularly.

10   The invention further describes how fusion molecules consisting of several domains can be constructed and successfully expressed and produced. The invention describes fusions of the targeting molecule to a small protein (CBD), to a moderately sized protein (EGI) and to a huge highly glycosylated protein (FloI), and different domain variations of these. These molecules can be ready as such for biotechnical use. Alternatively, the product can be
15   cleaved from the targeting protein by any method known in the art such as with proteases e.g. thrombin, factor X, papain or by chemical cleavage. Furthermore, ATPS is a preferential means to be used to separate the product from the targeting protein after cleavage, or these can be separated with other methods known in the art.

20   A suprising feature is that the targeting protein can also be used to carry large particles to the desired phase in ATPS. This can be obtained if the particles already contain proteins suited for targeting such as spores/conidia do in case of fungi. The targeting protein can also be attached to the particles or compounds in vitro. If cells are separated, the targeting protein can alternatively be expressed in the recombinant cells in such a way that it is
25   exposed at the cell surface whereby it mediates the separation of the cells in ATPS. A teaching how this can be done is provided in example 22. Other types of molecules which direct the targeting molecule to the cell surface can be found e.g. in the literature including bacterial outer membrane proteins and lipoproteins (Ståhl and Uhlén, 1997), and yeast proteins α-agglutinin and flocculin (Schreuder et al., 1996; Klis et al. (1994) WO 94/01567;
30   Frenken (1994) WO 94/18330).

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A further advantage of the system is that the invention combined with ATPS provides a means to separate the product or desired component not only from other unnecessary or unwanted proteins but also from harmful proteins such as proteases as described in example 6. Thus, the invention is particularly suited for production and purification of heterologous

5   proteins, e.g. sensitive mammalian proteins usually produced in limited amounts in heterologous hosts. Such proteins are for instance antibodies or fragments thereof, interferon, interleukin, oxidative enzymes and any foreign protein which can otherwise be produced in a host. It is possible that separation of the product from e.g. culture medium can also be obtained on-line or semi-continuously, thus minimising the effect of proteases

10   or other harmful components present in the culture. When produced intracellularly, the invention also provides means to separate the heterologous product, for instance the inclusion bodies it may form, from the cellular extracts.

This invention describes for the first time that fusion proteins containing hydrophobin-like

15   molecules can be made and produced in significant amounts despite the very particular properties of hydrophobin-like molecules. Importantly, this invention also describes how recombinant strains producing increased amounts of hydrophobin-like proteins as such can be made. This provides means to produce the targeting protein for uses in which it is wanted that the targeting protein is bound to the product or particle *in vitro*, to enable further

20   separation of such molecules or particles in ATPS.

Importantly, this invention also decsribes how hydrophobin-like molecules can be purified in ATPS very efficiently with high K-values. The molecules can be separated in the same way as the above mentioned fusions, for instance by PEG systems of by detergent-based

25   systems. Separation can be done from the culture medium or from cells. This provides a significant improvement in making pure preparates containing hydrophobin-like molecules since due to their properties their purification is very complicated and results in losses with the previously reported techniques as described above.

30   **Brief description of drawings**

Fig. 1   shows the map of the plasmid pMQ103.

Fig. 2   shows the map of the plasmid pMQ113.

Fig. 3   shows the map of the plasmid pMQ104.

5   Fig. 4   shows the map of the plasmid pMQ114.

Fig. 5   shows the map of the plasmid pMQ105.

Fig. 6   shows the map of the plasmid pMQ115.

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Fig. 7   shows the map of the plasmid pMQ121.

Fig. 8   Coomassie-stained 10% SDS-PAGE of the partitioning of EGIcore-HFBI
             fusion protein in two-phase separation using 5 % of the detergent C12-
15   C18EO5. Lane 1, Molecular weight marker; Lane 2, Purified CBHI (4 µg);
             Lane 3, Purified EGI (4 µg); Lane 4, 1/10 diluted VTT-D-98691 cellulose-
             based culture filtrate; Lanes 5 and 6, 1/10 diluted bottom phase and detergent
             phase (top phase), respectively, after separation of VTT-D-98691 culture
             filtrate with 5% detergent; Lane 7, Non-diluted bottom phase; Lane 8, Non-
20   diluted VTT-D-98691 cellulose culture filtrate.

Fig. 9   Western analysis of the partitioning of EGIcore-HFBI fusion protein in two-
             phase separation by using different concentrations of the detergent C12-
             C18EO5. Fusion proteins were detected with anti-HFBI antibodies. Lane 1,
25   Molecular weight marker; Lane 2, Purified EGI; Lane 3, VTT-D-98691
             cellulose culture filtrate; Lanes 4 and 5, Detergent phase (top phase) and
             bottom phase, respectively, after separation of VTT-D-98691 culture filtrate
             with 5% detergent; Lane 6, Same as lane 3, except 2 % detergent was used;
             Lane 7, Same as lane 4, except 2 % detergent was used; Lane 8, Purified EGI;
30   Lane 9:, Purified CBHI.

Fig. 10   Coomassie-stained 10% SDS-PAGE showing further purification of EGIcore-HFBI fusion protein from the endogenous CBHI when the top phase was re-extracted with 2% detergent. Lane 1, Molecular weight marker; Lane 2, Purified CBHI (4 µg); Lane 3, Purified EGI (4 µg); Lane 4, Detergent phase (top phase) after first extraction; Lane 5, Detergent phase (top phase) after second extraction.

Fig. 11   Coomassie-stained 10% SDS-PAGE analysis of the EGI-HFBI protein when treated with thrombin. Lane 1, Molecular weight marker; Lane 2, EGI-HFBI (1 mg/ml) treated 72 h with 3 U of thrombin at 24°C; Lane 3, Same as lane 2, except no thrombin was added; Lane 4, EGI-HFBI (1 mg/ml) treated 48 h with 9 U of thrombin at 36°C; Lane 5, Same as lane 4, except no thrombin was added; Lane 6, Same as lane 5, except no incubation at 36°C.

Fig. 12   shows the map of the plasmid pTNS13.

Fig. 13   Western analysis of the partitioning of dCBD-HFBI fusion protein in two-phase separation using 5 % of the detergent C12-C18EO5. Fusion protein was detected with anti-HFBI antibody. Lane 1, Four times concentrated culture filtrate; Lane 2, Four times concentrated bottom phase; Lane 3, Top phase.

Fig. 14   shows the map of the plasmid pTNS15. A non-functional restriction site is indicated with an asterisk.

Fig. 15   shows the map of the plasmid pTNS18.

Fig. 16   shows the map of the plasmid pTNS23.

Fig. 17   shows that separation to the detergent phase of cells expressing a hydrophobin on cell surface occurs: the top detergent phase of S. cerevisiae VTT-C-99315 is turbid, whereas the top detergent phase of the control strain H2155 is clear.

Fig. 18   shows the map of the plasmid pTNS32.

Fig. 19   shows the map of the plasmid pTH4.

5   Fig. 20   shows the map of the plasmid pTNS30.

Fig. 21   shows the map of the plasmid pTH1.

Fig. 22   shows the map of the plasmid pTH2.

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Fig. 23   shows the map of the plasmid pKS2.

Fig. 24   SDS gel showing the purification of HFBI-dCBD in a Berol 532 ATPS system.

15   Fig. 25   Western analysis of the partitioning of HFBI-ENA5ScFv fusion protein in two-phase separation using 4% of the detergent C12-C18EO5. Lanes from left: (1) Molecular weight marker, (2) Top (enriched) phase of the strain VTT-D-00791, (3) Bottom (depleted) phase of the strain VTT-D-00791.

20   Fig. 26   Western analysis of the partitioning of SC3 hydrophobin using SC3 specific antibodies. Lane 1, Bottom phase of the strain VTT-D-00792, Lane 2, Top phase of the strain VTT-D-00792.

Fig. 27   HPLC analysis for monitoring of the partitioning of HFBI in 2% Berol 532.

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Fig. 28   HPLC analysis for monitoring of the partitioning of HFBII in 2% Berol 532.

The invention is further illustrated by the following Examples which describe construction of the fusion molecules of the invention, and partitioning of the molecules of interest using

30   the process according to the invention.

EXAMPLES

Example 1

Construction of vectors for expression of EGI and EGIcore HFBI fusion proteins

5   **under the *cbh1* and *gpd1* promoters of *Trichoderma* and *gpdA* promoter of *Aspergillus***

For construction of an EGI-HFBI fusion protein, *hfb1* (SEQ ID 1) coding region (from Ser-23 to the STOP codon) and a peptide linker (Val Pro Arg Gly Ser Ser Ser Gly Thr Ala Pro Gly Gly) preceding it was amplified with PCR using pTNS9 as a template and as a 5' primer

10   **TCG GG <u>C ACT ACG TG</u> C CAG TAT AGC AAC GAC TAC TAC TCG CAA TGC** *CTT GTT CCG CGT GGC TCT AGT* TCT GGA ACC GCA (SEQ ID 2) and as a 3' primer TCG TAC <u>GGA TCC</u> TCA AGC ACC GAC GGC GGT (SEQ ID 3). pTNS9 has been decribed in detail in Example 19. The sequence in bold in the 5' primer encodes 16 C-terminal residues of EGI. The sequence in italics is a thrombin cleavage site and the underlined

15   CACTACGTG is a DraIII site. The underlined GGATCC in the 3' primer is a BamHI site. The 280 bp PCR fragment was purified from agarose gel and ligated to pGEM-T T/A vector (Promega) resulting in pMQ102.

For construction of an EGIcore-HFBI fusion protein, the *hfb1* coding region (as above) was

20   amplified with PCR using pTNS9 as a template and as a 5' primer **ACT ACA CGG AG <u>G</u> <u>AGC TC</u> G ACG ACT TCG AGC AGC CCG AGC TGC ACG CAG AGC AAC GGC AAC GGC** (SEQ ID 4) and as a 3' primer SEQ ID 3. The sequence in bold in the 5' primer encodes amino acids 410-425 in EGI and the underlined GAGCTC is a SacI site. The 260 bp PCR fragment was purified from agarose gel and ligated to pPCRII T/A vector (Invitrogen)

25   resulting in pMQ111.

In the next step *Trichoderma* expression vectors for production of EGI-HFBI and EGIcore-HFBI fusion proteins under the control of *cbh1* promoter and terminator sequences were constructed. The expression vector used as a backbone in the constructs is pPLE3 (Nakari *et*

30   *al.* (1994) WO 94/04673) which contains a pUC18 backbone, and carries the *cbh1* promoter (SEQ ID 5) inserted at the EcoRI site. The *cbh1* promoter is operably linked to the full length *egl1* cDNA (SEQ ID 6) coding sequence and to the *cbh1* transcriptional terminator (SEQ ID

7). The plasmid pMQ102 was digested with DraIII and BamHI and the released 280 bp fragment containing *hfb1* and linker sequences was purified from agarose gel and ligated to pPLE3 digested with DraIII and BamHI. The plasmid pMQ111 was digested with SacI and BamHI and the 260 bp fragment containing the *hfb1* sequence was ligated to pPLE3 digested

5 with SacI and BamHI. The resulting plasmids pMQ103 (Figure 1) and pMQ113 (Figure 2) carry the coding sequences for full-length EGI linked to HFBI via a peptide linker and for EGIcore linked to HFBI via its own linker region, respectively, under the control of *cbh1* promoter and terminator sequences.

10 *Trichoderma* expression vectors for production of EGI-HFBI and EGIcore-HFBI fusion proteins under the control of *gpd1* promoter and terminator sequences of *Trichoderma* and *gpdA* promoter and *trpC* terminator sequences of *Aspergillus* were constructed as follows. A SacII site was inserted inbetween the XbaI and PacI sites of pMV4 using as an adapter annealed primers TAA CCG CGG T (SEQ ID 8) and CTA GAC CGC GGT TAA T (SEQ

15 ID 9). The resulting plasmid is pMVQ. pMV4 contains a pNEB193 (New England Biolabs) backbone, and carries a 1.2 kb *Trichoderma gpd1* promoter (SEQ ID 10) and a 1.1 kb *gpd1* terminator (SEQ ID 11) inserted at SalI-XbaI and BamHI-AscI sites, respectively. The expression cassettes for EGI-HFBI and EGIcore-HFBI were released from pMQ103 and pMQ113 with SacII and BamHI, purified from agarose gel and ligated to pMVQ cut with

20 SacII and BamHI. The resulting plasmids pMQ104 (Figure 3) and pMQ114 (Figure 4) carry the EGI-HFBI and EGIcore-HFBI cassettes, respectively, under the control of *Trichoderma gpd1* transcriptional control sequences. Expression plasmids pMQ105 (Figure 5) and pMQ115 (Figure 6) containing EGI-HFBI and EGIcore-HFBI cassettes, respectively, operably linked to the *gpdA* promoter and *trpC* terminator of *Aspergillus* were constructed.

25 EGI-HFBI and EGIcore-HFBI cassettes were released from plasmids pMQ104 and pMQ114 with XbaI and BamHI, blunted with T4 DNA polymerase and ligated to NcoI digesteded and T4 DNA polymerase treated pAN52-1 (SEQ ID 12). pAN52-1 contains a pUC18 backbone, and carries a 2.3 kb *gpdA* promoter and a 0.7 kb *trpC* terminator sequences of *A. nidulans*.

Example 2

Construction of vectors for over-production of HFBI on cellulase-inducing and - repressing media

5   For over-expression of HFBI under *cbh1* promoter the protein coding region of *hfb1* was amplified with PCR using as a template pEA10 (Nakari-Setälä *et al.*, 1996). pEA10 carries a 5.8 kb genomic SalI fragment containing *hfb1* coding and flanking sequences. GTC AA C̲ **C̲G̲C̲ G̲G̲ A CTG CGC ATC ATG AAG TTC TTC GCC ATC** (SEQ ID 13) was used as a 5' primer in the PCR and as a 3' primer SEQ ID 3. The sequence in bold in the 5' primer is
10   21 bp of *cbh1* promoter adjacent to translational start site of the corresponding gene and the underlined CCGCGG is a KspI site. The obtained fragment of 430 bp was digested with KspI and BamHI and ligated to pMQ103 digested with KspI and BamHI. The resulting plasmid pMQ121 (Figure 7) carries the coding sequence of *hfb1* operably linked to *cbh1* transcriptional control sequences. pEA10 plasmid is used for over-production of HFBI in
15   cellulase-repressing conditions.

Example 3

Transformation of *Trichoderma* and purification of the EGI-HFBI and EGIcore-HFBI producing and HFBI over-producing clones

20

Trichoderma reesei strains QM9414 (VTT-D-74075) and Rut-C30 (VTT-D-86271) were co-transformed essentially as described (Penttilä *et al.*, 1987) using 3-13 µg of the plasmids pMQ103, pMQ113, pMQ104, pMQ114, pMQ105, pMQ115, pMQ121 and pEA10 and as the selection plasmids 1-3 µg pToC202, p3SR2 or pARO21. pToC202 (pUC19 backbone)
25   and p3SR2 (pBR322 backbone) plasmids carry 2.7 kb XbaI and 5 kb EcoRI-SalI genomic fragments of *A. nidulans*, respectively, containing the *amdS* gene (Hynes *et al.*, 1983; Tilburn *et al.*, 1983). pARO21 is essentially the same as pRLMex30 (Mach *et al.* 1994) and carries the *E. coli hph* gene operably linked to 730 bp of *pki1* promoter and 1 kb of *cbh2* terminator sequences of *T. reesei*. The Amd+ and Hyg+ transformants obtained were streaked three
30   times onto plates containing acetamide and hygromycin, respectively (Penttilä *et al.*, 1987). Thereafter spore suspensions were made from transformants grown on Potato Dextrose agar (Difco).

19

The production of the fusion proteins EGI-HFBI and EGIcore-HFBI and HFBI was tested by slot blotting or Western analysis with EGI and HFBI specific antibodies from shake flask or microtiter plate cultivations carried out in minimal medium supplemented with either glucose,

5   lactose or a mixture of Solka flock cellulose and/or spent grain and/or whey. The spore suspensions of the fusion protein producing clones were purified to single spore cultures on selection plates (containing either acetamide or hygromycin). To determine the best producers, production of the fusion proteins was analyzed again from these purified clones as described above.

10

T. reesei strains selected for further fermentor cultivations are VTT-D-98692 (pEA10), VTT-D-98492 (pMQ121), VTT-D-98693 (pMQ103), VTT-D-98691 (pMQ113), VTT-D-98681 (pMQ105) and VTT-D-98682 (pMQ115). These strains have QM9414 as the host strain. VTT-D-99702 (pMQ113) has Rut-C30 as the host strain.

15

Example 4
Cultivation of the EGI-HFBI and EGIcore-HFBI protein producing and HFBI over-producing *Trichoderma* strains

20   EGI-HFBI and EGIcore-HFBI fusions were produced under the *cbh1* promoter in a 15-litre fermenter using *T. reesei* strains VTT-D-98693 (pMQ103) and VTT-D-98691 (pMQ113), respectively. Strains were grown 5 days on minimal medium (Penttilä *et al.*, 1987) containing 4% Solka flock cellulose (James River Corporation, Berlin, NH) and 2 % spent grain (Primalco, Koskenkorva, Finland). EGIcore-HFBI was also produced in fermenter (15 l)

25   using the Rut-C30 strain VTT-D-99702 (pMQ113) with 4 % lactose medium. To induce the production of EGI-HFBI and EGIcore-HFBI fusions under *Aspergillus gpdA* promoter, *T. reesei* strains VTT-D-98681 (pMQ105) and VTT-D-98682 (pMQ115) were cultivated in 15-litre fermenter. Strains were grown 3 to 5 days on minimal medium supplemented with 2% glucose, 0.2% Peptone, and 0.1% Yeast Extract, and with glucose feed to maintain the

30   glucose concentration in the range of 1 to 3% throughout the cultivation. HFBI over-producing strain VTT-D-98692 (pEA10) was grown similarly in 15 l on glucose medium and the strain VTT-D-98492 (pQM121) over-producing HFBI under *cbh1* promoter was

cultivated for 7 days in 15-litre fermentor on medium containing 4% Solka flock and 2% spent grain. The control cultivations with the host strains of the transformants, QM9414 (VTT-D-74075) and Rut-C30 (VTT-D-86271), were carried out on media containing i) Solka flock cellulose and either spent grain or whey, ii) lactose and iii) glucose similarly as

5   described above.

When proper some *T. reesei* transformant strains and their host strains were also cultivated at 28°C in shake flasks for 5 to 6 days in 50 to 150 ml volume of *Trichoderma* minimal medium (Penttilä *et al.*, 1987) suplemented with either 3% Solka flock cellulose and 1% spent grain or

10   3-4% glucose with glucose feeding.

Example 5

Standard separation assays and analysis

15   If not otherwise stated the standard ATPS and subsequent analyses and calculations were carried out as explained in this example.

In general whole fermentation broth, supernatant (biomass separated by centrifugation or filtration) or purified proteins in buffer were separated in 10 ml graduated tubes. First

20   detergent was added into the tubes and the tubes were then filled to 10 mg with protein containing liquid. The amount of detergent in the tube was calculated in weight percent of detergents. After thorough mixing in an overhead shaker the separation took place by either gravity settling in a water bath at constant temperature or by centrifugation at constant temperature. The separation usually was performed at 30°C, the standard amount of

25   detergent used was 2-5% (w/v). After separation the volume ratio was noted and samples were taken from the lighter and heavier phase for analysis.

Two-phase separations were analysed qualitatively by using SDS-PAGE gels followed by visualization of the fusion proteins with Coomassie brilliant blue R-250 (Sigma) or Western

30   blotting. Polyclonal anti-HFBI antibody were used in Western analysis for detection of EGIcore-HFBI, EGI-HFBI and dCBD-HFBI proteins together with alkaline phosphatase conjugated anti-rabbit IgG (Bio-Rad). Alkaline phosphatase activity was detected

21

colorimetrically with BCIP (5-bromo-4-chloro-3-indolyl-phosphate) used in conjunction with NBT (nitro blue tetrazolium) (Promega).

Contaminating endogenous EGI, CBHI and EGIII in the top phase was tested with
5   appropriate antibodies. Acidic protease activity in the top and bottom phase was also tested using the SAP method (Food Chemicals Codex, p. 496-497, 1981), which is based on the 30 min enzymatic hydrolysis of a hemoglobin substrate. All reactions were performed at pH 4.7 and 40 °C. Unhydrolyzed substrate was precipitated with 14% TCA and removed by filtration. The released tyrosine and tryptophan was determined spectrophotometrically. Total
10   protein concentrations were determined by *Non-Interfering* Protein Assay™ (Geno Technology, Inc).

EGI activity was detected using 4-methylumbelliferyl-ß-D-cellobioside (MUC) (Sigma M 6018) as substrate (Van Tilbeurgh H. & Caeyssens M., 1985; Van Tilbeurgh *et.al.*, 1982).
15   EGI hydrolyses the ß-glycosidic bond and fluorogenic 4-methylumbelliferone is released, which can be measured using a fluorometer equipped with a 360 excitation filter and a 455 nm emission filter. CBHI also hydrolyses the substrate and it was inhibited by addition of cellobiose (C-7252, Sigma). EGI containing liquid was added in an appropriate dilution to a buffer containing 50 mM sodium acetate buffer (pH 5), 0.6 mM MUC and 4.6 mM
20   cellobiose. The mixture was heated to 50°C. The reaction was stopped after ten minutes using 2% Na_2CO_3, pH 10. Purified CBHI was detected using the same assay as for EGI without the addition of the inhibitor cellobiose.

The partition coefficient K was defined as the ratio of the measured concentrations or
25   activities in the top and bottom phase, respectively.
The Yield Y was defined as follows:

$$Y_T = \frac{1}{1 + \left[\dfrac{V_B}{V_T} \cdot \dfrac{1}{K}\right]}$$

30   where Y_T is the Yield of the top phase, V_B and V_T are the volumes of top and bottom phase, respectively. The Yield of the bottom phase can be described accordingly.

The mass balances, e.g. recovery of all added protein, were always checked for completeness to ensure no artificially high Yield (e.g. due to possible inactivation of the protein in the bottom phase). The values were usually calculated based on total enzyme activity (EGI wt plus the EGI-fusion) and thus the values are underestimated for the separation of the fusion as

5   demonstrated in Example 16.

Example 6

Small scale ATPS separation studies and gel analysis

10   EGI-HFBI and EGIcore-HFBI fusions produced under the *cbh1* promoter in a 15-litre fermenter on Solka flock cellulose and spent grain medium as described in Example 4 using *T. reesei* strains VTT-D-98693 (pMQ103) and VTT-D-98691 (pMQ113), respectively, were separated in small scale ATPS as described above.

15   The phases from the two phase separations were analysed qualitatively by using SDS-PAGE gels followed by visualization of the fusion proteins with Coomassie brilliant blue or Western blotting. Coomassie stained SDS-PAGE (10%) is shown in Figure 8. In the lane containing the non-extracted culture filtrate three distinct closely migrating bands can be seen (the sample was diluted 1/10 with H_2O). The topmost band is CBHI, the band in the middle is

20   EGIcore-HFBI fusion and the lower one endogenous EGI. In the samples separated in ATPS, only two bands (CBHI and EGI) are seen in the sample from bottom phase and one band representing EGIcore-HFBI in the sample obtained from the top phase.

Western blotting with HFBI antibody showed thick bands for the top phase, whereas for the

25   bottom phase there was only faint band demonstrating that the fusion is separating strongly into the detergent top phase. Figure 9 shows the separation of the EGIcore-HFBI fusion produced on cellulose media into the top phase. Contaminating endogenous EGI and EGIII in the top phase was tested with appropriate antibodies but no signal was detected.

30   Small amount of endogenous CBHI was found in the upper phase when CBHI antibody was used in Western blotting. EGI, EGIII and proteases were not found in the top phase. Further purification from the contaminating CBHI was observed when the top phase was re-extracted

23

with 2 % detergent. The Figure 10 shows that the upper phase does not any more contain CBHI and pure fusion protein is recovered.

EGIcore-HFBI was also produced in fermenter (15 l) using the Rut-C30 strain VTT-D-
5   99702 (pMQ113) with 4 % lactose medium. The separation in ATPS carried out in the standard manner gave essentially the same result as the separation from cellulose containing medium thus demonstrating that the purification can be carried out from several media relevant for large scale industrial use.

10   Acidic protease activity in the top was only 1/15 compared to the bottom phase (table below) demonstrating that acidic proteases remain in the bottom phase.

	A (275 nm)	HUT^3/ml
Bottom phase^1	0.146	41.6
Top phase^2	0.009	2.6

[1] 1/10 diluted bottom phase after separation of VTT-D-98691 culture filtrate with 2% detergent
15   [2] 1/100 diluted bottom phase after separation of VTT-D-98691 culture filtrate with 2% detergent
[3] 1 HUT = enzyme concentration, which in reaction conditions hydrolyses hemoglobin in 1 min so that the absorbance at 275 nm of the formed hydrolysate equals 1.10 µg tyrosine/ml 0.006 N HCl solution.

These results show that the fusion protein can be purified extremely efficiently and the
20   resulting preparate is free of other proteins produced by the fungus including proteases.

Example 7
Recovery of the native EGI in ATPS after thrombin cleavage

25   EGI-HFBI protein produced by the strain VTT-D-98693 has a thrombin cleavage site (LVPRGS) designed in the linker region between the EGI CBD and HFBI, which would enable the recovery of the native EGI after thrombin cleavage. EGI-HFBI fusion protein was purified from the culture filtrate (100 ml) of strain VTT-D-98693 grown on 4% Solka flock

cellulose and 2 % spent grain as described in Example 4 using the 2-phase separation system (5 % detergent). After removal of the bottom phase the detergent phase was extracted by isobutanol. The resulting water phase (~19 ml) was divided in eppendorf tubes and the liquid was evaporated with speed vac. Remaining lyophilizate was diluted to 50 mM Tris-Cl (pH 8).

5   To test the efficiency of thrombin cleavage, 9 units of thrombin (Sigma) was incubated > 24 h with 1 mg EGI-HFBI fusion protein in 36 °C at pH 8.0. Coomassie stained SDS-PAGE (10 %) was used for detection.

Only minor cleavage was observed in 48 h under these conditions (Figure 11), possibly due to
10   steric hindrance by O-glycosylation in the linker.

Example 8

Separation of low concentrations of EGIcore-HFBI in ATPS

15   Detergent based aqueous two-phase systems were successfully applied using very low concentrations (diluted) of EGIcore-HFBI fusion protein produced with the *cbhl* promoter in *T. reesei* VTT-D-98691 (pMQ 113) from a 15 liter cultivation carried out on Solka flock cellulose with spent grain as described in Example 4.

20   The original protein concentration of the supernatant was 7.0 mg/ml. This supernatant was diluted with de-ionised water by a factor of 100 and 1000, respectively. The fusion protein could be separated using 2% (w/w) of the detergent C12-C18EO5 with partitioning coefficients higher than 5. This is shown in the table below together with the experiment with non-diluted supernatant. The partitioning coefficients were calculated based on activity
25   measurements for total EGI (wild type and fusion protein together).

	EGIcore-HFBI non-diluted supernatant	dilution 1/100	dilution 1/1000
K	4.1	5.3	5.6
Y [%]	38	31	32

Example 9

Separation of EGIcore-HFBI from fungal biomass containing culture broths

EGIcore-HFBI from *T.reesei* strain VTT-D-98691 (pMQ 113-2) was cultivated (50 ml in
5   250 ml shake flasks) on Solka flock cellulose with spent grain as described in Example 4.
Directly after the cultivation, part of the whole broth was centrifuged at 3000 rpm for 30 min,
supernatant was spilled out and the centrifuged mycelium was added to the supernatant to
obtain artificial whole broths containing different amounts of biomass.

10 Using 5% of C12-C18EO5 in a 10 g experiment consisting of up to 50% wet biomass
(weight of wet biomass divided by the sum of wet biomass and supernatant) could still be
separated without any difficulties. The Yield remained in between 61 and 64 % and therefore
it is not significantly different in comparison to the experiment carried out with supernatant
only (without mycelium) (see table below). The total recovery of the fusion protein is even
15 higher. This is most probably due to cell attached enzyme extracted in the ATPS increasing
the total amount of EGI. The partitioning coefficient was calculated based on activity
measurements for total EGI (wild type and fusion protein together).

	K	Y[%]
Supernatant	5.5	62
25% of wet biomass in supernatant	7.3	66
40% of wet biomass in supernatant	6.4	61
50% of wet biomass in supernatant	7.6	64

Example 10

Separation of EGI-HFBI in ATPS

EGI-HFBI from *Trichoderma reesei* strain VTT-D-98693 (pMQ 103) from a 15 liter
5   cultivation carried out on Solka flock cellulose and spent grain as described in Example 4 was
separated in a 10 g experiment using different amounts of C12-C18EO5. The partitioning
coefficients are shown below. The partitioning coefficient was calculated based on activity
measurements for total EG1 (wild type and fusion protein together), and as in previous
examples the endogenous EGI is included in the partitioning coefficients.

10

Detergent [% w/w]	2	3	5	7
K	1.9	1.8	1.4	1.1

Example 11

Separation of EGIcore-HFBI in 50 ml

15

EGIcore-HFBI from *T. reesei* strain VTT-D-98691 (pMQ 113) cultivated in 15 liters using
Solka flock cellulose and spent grain as described in Example 4 was separated in Falcon tubes
in a 50 g experiment using 5% of C12-C18EO5. A partition coefficient of 2.52 and a yield of
51 % could be obtained. The separation was performed at 30°C at 3000rpm for 30 minutes.
20   The values are based on activity measurements for total EGI activity (wild type and fusion
protein together) including endogenous EGI.

Example 12

Separation of EGIcore-HFBI in ATPS using different detergents

25

EGIcore-HFBI from *T. reesei* strain VTT-D-98691 (pMQ 113) cultivated in 15 liters using
Solka flock cellulose with spent grain as described in Example 4 was separated in a 10 g
experiment using 2% of detergent in each experiment. The detergents investigated in this
example were C10 EO5, C12 EO5, C14 EO6 (each Nikko Chemicals, Japan), C12-C18 EO5

("Agrimul NRE 1205", Henkel, Germany), C12/14 5EO, C12/14 6EO (Clariant, Germany), C9/11 EO5.5 ("Berrol 266", Akzo Nobel, Germany), Triton X-114 (Sigma, Germany). The partition coefficients and yields are listed below. The values are based on activity measurements for total EGI activity (wild type and fusion protein together) including

5   endogenous EGI.

	K	Y(fusion) [%]
C10EO5	20	56
C12EO5	15	57
C12-C18EO5	14	66
C12/14 5EO	12	58
C12/14 6EO	14	62
C14EO6	11	54
C9/11 EO5.5	5	30
Triton X-114	0.16	53

10   **Example 13**

Separation of EGIcore-HFBI in ATPS from glucose grown cultures

EGIcore-HFBI was separated from cultivation of the *Trichoderma reesei* strain VTT-D-98682 (pMQ115) cultivated with glucose as described in Example 4. The supernatant was

15   separated with 2% of the detergent C12-C18 EO5. The fusion protein could be partitioned with a K value of 2.4. In comparison, the K value for the native EGI is 0.3 when measured in a similar way for purified EGI.

Example 14

20   **Separation of EGIcore-HFBI using different concentrations of detergent**

EGIcore-HFBI from *T. reesei* VTT-D-98691 (pMQ 113) cultivated in 15 litres using Solka flock with spent grain as described in Example 4 was separated in detergent based ATPS

applying different amounts of the detergent C12-C18 EO5 on the cell free supernatant. The partitioning coefficients are shown in the table below. The corresponding gel electrophoresis and Western antibody-blots are shown in Figure 8 and Figure 9, respectively.

The values are based on activity measurements of total EGI activity.

5

Amount of detergent C12-C18 EO5	K	Yield(%)
1.0%	6.1	9
2.0%	4.1	38
3.5%	3.6	50
5.0%	2.9	55
7.5%	1.7	53
10.0%	1.1	58

Example 15

Re-extraction of the detergent phase

10

Detergent based ATPS was applied on EGIcore-HFB1 fusion protein containing supernatant produced by the strain VTT-D-98691 (pMQ 113) in a shake flask cultivation. The first extraction using C12-C18EO5 conducted under the standard conditions shows a partitioning coefficient of 16 and a yield of 72 % (wild type EGI measured together with fusion protein).

15 The top phase was re-extracted in 10 mM sodium acetate buffer (pH 5) with 2% of detergent. A partitioning coefficient of 52 and a yield of 89 % could be obtained. In the re-extraction experiment of the bottom phase (2% of detergent), a small yield of 7.5% and a K of 0.8 of EGI activity were achieved. The partitioning coefficients were calculated based on activity measurements for total EGI (wild type and fusion protein together). Due to the wild

20 type EGI present in the sample, the yield is at least 72% and the partitioning coefficient at least 16 in the first extraction. The SDS-PAGE results of both extraxtions are shown in Figure 10.

Separation step	K	Y[%]
2% detergent	16	72
reextraction top phase	52	89
reextraction bottom phase	0.8	7.5

Example 16

Separation of pure cellulases in ATPS

The effect of HFBI on partitioning and the final yield can further be demonstrated by comparing the extraction result of EGIcore-HFB fusion with extraction results obtained with purified wild type EGI and EGIcore. The fusion protein is partitioning more than 100 times better to the detergent phase (see table belove).

The improvement on the partitioning of the purified fusion protein from the first extraction obtained in the re-extraction (see Example 15) can be explaned by the partitioning of the wild type EGI as demonstrated with purified wild type EGI in the table below. The wild type EGI lowers the partitioning coefficient in the first extraction (since EGI activity is measured from both top and botom phase), but the absence of it in the re-extraction increases the partitioning coefficient of the EGIcore-HFBI fusion. The purity can in addition be demonstrated by analysing the partitioning of pure CBHI, which is the major contaminating protein corresponding to about 50 % of all secreted *T. reesei* proteins. Pure CBHI has a partitioning coefficient of 0.5 and a yield of 3.6 and is therefore separated from the fusion protein.

Separation step	K	Y[%]
re-extraction of top phase	52	89
extraction of pure wild type EGI	0.3	2.2
extraction of pure EGI-core	0.3	2.3
extraction of pure CBHI	0.5	3.6

Using the definitions of K and Y and calculating mass balances, the ratio of the amount of EGI fusion protein to EGI wild type can be calculated. The "true" partition coefficients and Yields can be concluded from this. "True" means the values which would be detected if it would be possible to measure only the amount of EGI-fusion without measuring the amount

5   for EGI wild type at the same time.

The fundament for the calculation is the re-extraction experiment. The re-extracted top phase is believed to be pure. An example of the measured values and the calculated "true" values based on this are shown in the table below for two cultivations of VTT-D-98691 (pMQ113)

10   grown as described in Example 4.

cultivation vessel	cultivation substrate	K "with EGl wt"	"true" K	Y [%] "with EG1 wt"	"true" Y [%]
15 liter fermenter	whey permeate	4	6	16	54
250 ml shake flask	cellulose	16	54	66	90

15   **Example 17**

HFBI and HFBII purification in ATPS

HFBI was produced by cultivating the *T. reesei* strain VTT-D-98692 (pEA10-103B) using glucose as substrate as described in Example 4. HFBI could be separated using 2% of the

20   detergent C12-C18 EO5 with a partition coefficient higher than 20 under the standard conditions described.

HFBII was produced by cultivating the *T. reesei* strain VTT-D-74075 (QM9414) on whey spent grain as described in Example 4. HFBII could be separated using 2% of the detergent

25   C12-C18 EO5, exceeding a partition coefficient of 10 under the standard conditions.

Both HFBI and HFBII hydrophobins are thus partitioning well to the upper phase in ATPS.

Example 18

Detergent based ATPS with additional NaCl

5   EGIcore-HFBI from which cultivation of T.reesei was separated in a 10 g experiment using 5% of C12-C18EO5. The partitioning coefficient of the supernatant was 3.5 with a volume ratio of 0.2. Using 1.1 % (w/v) NaCl the partitioning coefficient could be increased to 4.3 with a lower volume ratio of 0.14.

Example 19

10   ***Construction of an E. coli strain expressing a fusion protein HFBI-dCBD, containing hydrophobin I and double cellulose binding (CBD) domains***

A 280 bp DNA fragment containing a modified *cbh2* linker region followed by the coding region of *hfb1* from Ser-23 to the STOP codon was amplified by PCR using the plasmid

15   pARO1 (Nakari-Setälä *et al.*, 1996) as a template. The 5' primer was 5' TCT AGC <u>AAG CTT</u> **GGC TCT AGT TCT GGA ACC GCA CCA GGC GGC** AGC AAC GGC AAC GGC AAT GTT TGC (SEQ ID 14) and the 3' primer was 5' TCG TAC <u>AAG CTT</u> TCA AGC ACC GAC GGC GGT (SEQ ID 15). The sequences in bold in the 5' and 3' primers encode the modified CBHII linker (Gly Ser Ser Ser Gly Thr Ala Pro Gly Gly) and a

20   translational STOP, respectively, and the underlined AAGCTT in both primers is a HindIII site. The PCR fragment was purified from agarose gel, digested with HindIII and ligated to HindIII digested and SAP treated (Shrimp Alkaline Phosphatase, USB) pSP73 resulting in plasmid pTNS9.

25   For subsequent cloning of the modified CBHII linker-HFBI fragment to an *E. coli* expression vector, pTNS9 was digested with HindIII and the proper fragment was purified from agarose gel. This HindIII fragment was cloned to HindIII digested and SAP treated (Shrimp Alkaline Phosphatase, USB) B599 resulting in pTNS13 (Figure 12). The *E. coli* expression vector B599 is essentially the same as the one described by Linder *et al.* (1996) except that it is

30   missing a STOP codon at the end of the protein coding sequence. It carries the coding sequence for a fusion protein containing CBHII CBD (41 N-terminal residues of CBHII) and CBHI CBD linked together via CBHI linker region (CBHI linker and CBD are the last 57

residues in CBHI). The expression and secretion of the fusion protein in B599 is under the cotrol of *tac* promoter and *pelB* signal sequence (Takkinen *et al.*, (1991). pTNS13 expression vector thus carries the coding region for a fusion protein of double CBD and HFBI linked in frame via the Gly-Ser-Ser-Ser-Gly-Thr-Ala-Pro-Gly-Gly peptide. This vector also contains

5   the *amp* gene for selection of *E. coli* transformants. pTNS13 plasmid was transformed into *E. coli* strain RV308 (su-, Δ*lac*X74, *gal*ISII::OP308, *str*A) and this strain was used for production of the fusion protein.

Example 20

10   **Separation of HFBI-dCDB molecules expressed in *E.coli* in ATPS**

dCBD-HFBI was produced in *E. coli* strain RV 308 transformed with pTNS13 plasmid as described above. The inoculum of RV308/pTNS13 was grown to the exponential growth phase in LB medium containing ampicillin (0.1 g/l) and 1% glucose. Fermentation was carried

15   out using mineral salt medium described by Pack *et al.* (1993) with glucose (feed) in 10 litre fermenter. During cultivation temperature was maintained at 28 °C and pH was controlled at 6.8 with NH$_4$OH. Cell growth was monitored by measuring OD$_{600}$ and dry weight of biomass. The culture was induced with 50 µM (final concentration) IPTG (isopropyl-β-D-thiogalactopyranoside) at late-exponential growth phase (OD$_{600}$=50-60) to promote fusion

20   protein production.

Two-phase separation analysis of dCBD-HFBI protein was performed using culture filtrate and 5% detergent in the total volume of 40 ml. Results from Western blotting showed that 2-phase separation with 5 % detergent in the standard way was highly specific also for the

25   dCBD-HFBI fusion. Strong signal was observed in the sample from the detergent phase compared to the sample from the bottom phase as shown in Figure 13.

Example 21

Construction of yeast strains expressing HFBI-Flol fusion protein on the cell surface

For construction of a HFBI-FLO1 fusion protein expression cassette, *hfb1* (SEQ ID 1)

5   coding region (from Ser-23 to the STOP codon) was amplified with PCR using pARO1
(Nakari-Setälä *et al.*, 1996) as a template and as a 5' primer TCT AGC TCT AGA AGC
AAC GGC AAC GGC AAT GTT (SEQ ID 16) and as a 3' primer TGC TAG TCG ACC
TGC TAG CAG CAC CGA CGG CGG TCT G (SEQ ID 17). The underlined sequences in
the 5' and 3' primers are XbaI and NheI sites, respectively. The 0.225 bp PCR fragment was

10   purified from agarose gel and ligated to pGEM-T vector (Promega) resulting in pTNS10.
The *hfb1* fragment was released from pTNS10 with XbaI and NheI and ligated to pTNS15
cut with the same restriction enzymes. Plasmid pTNS15 (Figure 14) is essentially the same as
plasmid pBR-ADH1-FLO1L by Watari *et al.* 1994 except that a NheI site in the pBR322
backbone has been replaced by a BglII site and a unique XbaI site is introduced by linker

15   cloning in the unique AocI site preceding the putative signal sequence cleavage site. The
resulting plasmid pTNS18 (Figure 15) contains the complete expression cassette for HFBI-
FLO1 fusion protein in which HFBI substitutes the putative lectin domain from Ser-26 to
Ser-319 in the yeast flocculin FLO1 (SEQ ID 18).

20   In the next step, yeast expression vector for production of HFBI-FLO1 fusion protein was
constructed. The expression vector used as a backbone in the construct is pYES2
(Invitrogen) (SEQ ID 19) which is a high-copy episomal vector designed for inducible
expression of recombinant proteins in *S. cerevisiae*. It carries *GAL1* promoter and *CYC1*
terminator sequences which regulate transcription, and 2μ origin of replication and *URA3*

25   gene for maintenance and selection in the host strain. The plasmid pTNS18 was digested with
HindIII and the released 3.95 kb fragment containing the expression cassette for HFBI-FLO1
was purified from agarose gel and ligated to pYES2 digested with HindIII. This ligation
mixture was concentrated by standard ethanol precipitation. The ligation mixture should
contain besides unligated fragments and uncorrect ligation products also molecules where the

30   vector and insert are correctly ligated with each other to result in plasmid pTNS23 (Fig. 16)
which carries the expression cassette for HFBI-FLO1 operably linked to *GAL1* and *CYC1*
terminator sequences.

The above ligation mixture was transformed using the LiAc method of Gietz *et al.* (1992) into a laboratory *S. cerevisiae* strain H452 (wild type W303-1A; Thomas and Rothstein, 1989). Transformant colonies able to grow on SC-URA plates were picked and streaked on

5   selective plates. Nitrocellulose replicas were taken from the plates and treated for colony hybridization according to Sherman *et al.* (1983). To find those yeast colonies containing the pTNS23 plasmid, replicas were hybridized with digoxigenin labelled *hfbI* coding fragment after which an immunological detection was performed all according to the manufacturer (Boehringer Mannheim). Plasmids were recovered from several yeast colonies giving positivie

10   hybridization signal by isolating total DNA and using this in electroporation of *E. coli*. Restriction mapping and sequencing were carried out to confirm that the pTNS23 plasmid in the yeast transformants was correct. One of the transformants carrying plasmid pTNS23 was chosen for further studies and was designated VTT-C-99315. The control strain for it is yeast strain H2155 which carries the plasmid pYES2 in H452 background.

15

Example 22

Separation of yeast cells expressing HFBI-Flo1 fusion protein in ATPS

The *Saccharomyces cerevisiae* strain VTT-C-99315 (vector pTNS23) and its control strain

20   H2155 (vector pYES2) were cultivated on synthetic complete medium lacking uracil (SC-URA) (Sherman, 1991) with 2% galactose as the carbon source to give an A_{600} of approximately 4. Approximately 6.3×10^8 cells in their culture medium were taken to ATPS using 7% (w/v) C12-18EO5 detergent (Agrimul NRE from Henkel) in a total volume of 5 ml. ATPS was carried out using strandard protocol. After phase separation by gravity settling,

25   the top detergent phase was clearly turbid in the case of the strain VTT-C-99315 in contrast to the control strain whose detergent phase was clear (Figure 17). Samples were taken from the top phases and dilution series from 10^{-1} to 10^{-5} were prepared in 0.9% NaCl and plated on YPD plates. After incubation at 30°C the amount of yeast colonies were calculated showing at least 70 times more yeast colonies of the strain VTT-C-99318 on YPD plates compared to

30   the control strain. This clearly manifests that also in a system over-loaded with cells, separation to the detergent phase of cells expressing a hydrophobin on cell surface occurs.

35

Example 23

Partitioning of EGIcore-HFBI fusion protein in hydrophobin-containing and non-containing pure systems

5   The effect of free HFBI and HFBII hydrophobins on the partitioning of EGIcore-HFBI was investigated by comparing how efficiently a sample of purified EGIcore-HFBI in 50 mM acetate buffer pH 5.0 was extracted to the detergent phase in the presence and absence of purified HFBI and HFBII. The extraction was followed by measuring the depletion of hydrolytic activity on a soluble substrate such as methylumbelliferyl

10   cellobioside in the aqueous phase before and after extraction. When 0.7 g/l of either purified hydrophobin was present in the separation of 0.02 g/l of EGIcore-HFBI with 2% C12-18EO5 at 30°C the extraction was affected in the following way: 93% of the protein was extracted when additional hydrophobin was not present, but with HFBII present 82% was extracted and with HFBI present 88% was extracted.

15

Example 24

Production of EGIcore-HFBI fusion proteins in *T. reesei* Δ*hfb2* strain for improved *partitioning of the fusion protein in ATPS*

20   *Trichoderma reesei* strain QM9414 Δ*hfb2* (VTT-D-99726) was transformed essentially as described (Penttilä *et al.*, 1987) using 10 µg of the plasmid pMQ113 (described in Example 1) together with 3 µg of the selection plasmid pTOC202 containing the *amdS* gene (Hynes *et al.*, 1983); Tilburn *et al.*, 1983) of *Aspergillus nidulans* encoding for acetamidase. pMQ113 contains an expression cassette for production of EGIcore-HFBI fusion protein under the

25   control of *cbh1* promoter and terminator sequences.

The Amd+ transformants obtained were streaked two times onto plates containing acetamide (Penttilä *et al.*, 1987). Thereafter spore suspensions were made from transformants grown on Potato Dextrose agar (Difco). The production of the EGIcore-HFBI fusion protein was

30   tested by slot blotting or Western analysis with EGI and HFBI specific antibodies from shake flask or microtiter plate cultivations carried out in minimal medium supplemented with Solka

flock cellulose. The spore suspensions of the clones producing fusion protein were purified to single spore cultures on selection plates containing acetamide. To determine the best producers, production of the fusion protein was analyzed again from these purified clones as described above.

5

For partitioning experiments of the EGIcore-HFBI fusion protein in ATPS using the polyoxyethylene detergent C12-18EO5 the best production strain obtained in this study and as control strains VTT-D-98691 (QM9414 strain producing EGIcore-HFBI) and VTT-D-74075 (QM9414) were cultivated in shake flasks on medium containing Solka flock cellulose
10 as described in Example 4.

Standard partitioning experiments as described in Example 5 are carried out with culture supernatants. After separation the volume ratio of the lighter and heavier phase is noted and the concentration factor for the fusion protein is calculated from it. Samples are also taken
15 from the lighter and heavier phase and analysed with SDS-PAGE, Western blotting and activity measurements as described in Example 5. Partition coefficients (K) and yields (Y) are calculated as described in Example 5.

Example 25
20 **Construction of vectors for expression of EGIcore-HFBII and EGIcore-SC3 fusion proteins under the *cbh1* promoter of *T. reesei***

For construction of an expression vector for production of EGIcore-HFBII fusion protein, *hfb2* (SEQ ID 20) coding region (from Ala-16 to the STOP codon) was amplified by PCR
25 using phfb2 (Nakari-Setälä et al. 1997) as a template and as a 5' primer CGG AG**G AGC TC**G ACG ACT TCG AGC AGC CCG AGC TGC ACG CAG GCT GTC TGC CCT ACC GG (SEQ ID 21) and as a 3' primer TCA TT**G GAT CC**T TAG AAG GTG CCG ATG GC (SEQ ID 22). The sequence is bold in the 5' primer encodes for amino acids 413-425 of EGI and the underlined GAGCTC is a SacI site. The underlined GGATCC in the 3'
30 primer is a BamHI site. The amplified fragment was digested with SacI and BamHI and ligated to pMQ113 cut with the same restriction enzymes. The resulting plasmid is pTNS32

(Figure 18) and it carries the coding sequence for EGIcore-HFBII fusion protein under the control of *cbh1* regulatory sequences (SEQ ID 5 and SEQ ID 7).

For construction of an expression vector for production of EGIcore-SC3 fusion protein, SC3 cDNA (SEQ ID 23) was amplified with PCR using cSC3/pUC20 plasmid as a template and as a 5' primer ACT ACA CGG A**GG AGC TC**G ACG ACT TCG AGC AGC CCG AGC TGC ACG CAG GGT GGC CAC CCG GGC (SEQ ID 24) and as a 3' primer TCG TAC GGA TCC TCA GAG GAT GTT GAT GGG (SEQ ID 25). The sequence is bold in the 5' primer encodes for amino acids 413-425 of EGI and the underlined GAGCTC is a SacI site. The underlined GGATCC in the 3' primer is a BamHI site. The amplified fragment was digested with SacI and BamHI and ligated to pMQ103 (described in Example 1) cut with the same restriction enzymes. The resulting plasmid is pTH4 (Figure 19) and it carries the coding sequence for EGIcore-SC3 fusion protein under the control of *cbh1* regulatory sequences (SEQ ID 5 and SEQ ID 7).

The cSC3/pUC20 plasmid contains the 411 bp SC3 cDNA from the translational start site to the STOP codon in a pUC20 vector. The translational start site has been constructed to a NcoI site and a BamHI site has been added after the translational STOP codon.

Example 26

Construction of a vector for expression of a HFBI-dCBD fusion protein containing hydrophobin I and double cellulose binding domains (CBD) under the *cbh1* promoter of *T. reesei*

For construction of an expression cassette for production of HFBI -dCBD fusion protein under *cbh1* promoter, the protein coding region of *hfb1* was amplified with PCR using pEA10 (Nakari-Setälä et al. Eur. J. Biochem. (1996) 235:248-255) as a template. GGA ATT **CCG CGG** ACT GCG CAT CAT GAA GTT CTT CGC CAT CGC C (SEQ ID 26) was used as a 5' primer in the PCR and TGA ATT C*CA TAT G*TT A*GG TAC CAC CGG GGC CCA TGC CGG TAG AAG TAG AAG CCC CGG G*AG CAC CGA CGG CGG TCT GGC AC (SEQ ID 27) as a 3' primer. The sequence in bold in the 5' primer is 16 bp of *cbh1* promoter adjacent to translational start site of the corresponding gene

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and the underlined CCGCGG is a KspI site. The underlined and bold sequences in the 3'
primer are NdeI and Asp718 sites, respectively. The sequence in Italics in the 3' primer
encodes for a Methionine-containing linker (PGASTSTGMGPGG). The resulting
fragment of 370 bp was digested with KspI and NdeI and ligated to pAMH110

5   (Nevalainen, K.M.H., Penttilä, M.E., Harkki, A., Teeri, T.T. and Knowles, J. (1991) In
Molecular Industrial Mycology. Eds. Leong, S.A. and Berka, R.. Marcel Dekker. New
York) digested with same restriction enzymes. The resulting plasmid is pTNS29-2Asp.

For further cloning steps, an Asp718 site was removed from the pTNS29-2Asp
10   polylinker. The vector was digested with SacI and BamHI and the cleaved vector ends
were blunted with T4 DNA polymerase and ligated together. The resulting vector
pTNS29 is missing the SacI, Asp718 and SmaI sites present in the pTNS29-2Asp
polylinker.

15   In the next step, a double cellose binding domain (dCBD) was amplified with PCR using
pTNS11 as a template. pTNS11 carries the coding sequence for a fusion protein
containing *T. reesei* CBHII CBD (41 N-terminal residues of CBHII) and CBHI CBD
linked together via CBHI linker region (CBHI linker and CBD are the last 57 residues in
CBHI). This DNA sequence originates from an *E. coli* expression vector B599 which is
20   essentially the same as the one described by Linder et al. (J. Biol. Chem. (1996)
271:21268-21272). TGA ATT CGG TAC CCA GGC TTG CTC AAG CGT C (SEQ ID
28) was used as a 5' primer in the PCR and TGA ATT CCA TAT GTC ACA GGC ACT
GAG AGT AGT A (SEQ ID 29) as a 3' primer. The underlined sequences in the 5' and 3'
primers are Asp718 and NdeI sites, respectively. The amplified fragment was digested
25   with Asp718 and NdeI and ligated to pTNS29 digested with Asp718 and NdeI resulting
in pTNS30.

pTNS30 (Figure 20) expression vector thus carries the coding region for a fusion protein
consisting of HFBI and double CBD linked in frame via the Methionine linker peptide
30   (PGASTSTGMGPGG). Expression of the fusion protein is regulated by the *cbh1*
transcriptional control sequences. The expression cassette may be released from the
plasmid with EcoRI and SphI.

Example 27

Construction of vectors for expression of HFBI-single chain antibody fusion proteins under the *cbh1* promoter of *T. reesei*

5   An expression vector was constructed for production of a fusion protein consisting of *T. reesei* HFBI protein in the N-terminus and in the C-terminus a single chain antibody recognizing a small molecular weight derivative of diarylalkyltriazole (ENA5ScFv). Production of the fusion protein is under the *cbh1* regulatory sequences. For construction of HFBI-ENA5ScFv fusion protein, pENA5ScFv was digested with NcoI and XbaI. The

10   fragment containing the *ena5scfv* gene and the histidine tail (6 x His) was blut-end cloned to pTNS29 resulting in pTH1 (Figure 21). pENA5ScFv vector carries the coding region for ENA5 single chain antibody consisting of the variable domains of the heavy and light chains connected via a glycine serine linker and a 6 x histidine tag at the C-terminal end. Transcription and secretion of the single chain antibody are under control of the *tac* promoter

15   and *pelB* signal sequence, respectively (Takkinen *et al.*, 1991). pTNS29 vector carries the *hfb1* coding region of *T. reesei* followed by a linker sequence (ProGlyAlaSerThrSerThrGlyMetGlyProGlyGly) under the control of *cbh1* promoter and terminator sequences.

20   For construction of HFBI-ENA5ScFv fusion protein with a thrombin cleavage site in the linker peptide, *ena5scfv* coding region (from Ala-23 to the STOP codon) and a peptide linker containing the thrombin cleavage site (Gly Thr Leu Val Pro Arg Gly Pro Ala Glu Val Asn Leu Val) preceeding it was amplified with PCR using pENA5ScFv as a template and as a 5' primer GAA TTC *GGT ACC CTC GTC CCT CGC GGT* CCC **GCC GAA GTG AAC**

25   **CTG GTG** (SEQ ID 30) and as a 3' primer TGA ATT C<u>CA TAT G</u>CT AAC CCC GTT TCA TCT CCA G (SEQ ID 31). The sequence in bold in the 5' primer encodes the first 6 N-terminal residues of ENA5SCFV. The sequence in italics is a thrombin cleavage site and underlined GGT ACC is an Asp718 site. The sequence in bold in the 3' primer encodes the 6 C-terminal residues of ENA5ScFv and the underlined CA TATG is a NdeI site. The 790 bp

30   PCR fragment was purified from agarose gel and ligated to pTNS29 resulting in pTH2 (Figure 22).

Example 28

Construction of a vector for production of class I hydrophobin SC3 of *Schizophyllum commune* in *Trichoderma reesei* for ATPS

5   A *T. reesei* strain producing class I hydrophobin SC3 of *S. commune* was constructed. For that purpose an expression vector carrying the SC3 cDNA under the control of *hfb2* promoter and *hfb1* terminator of *T. reesei* was constructed.

The *hfb1* terminator (SEQ ID 32) was amplified with PCR using as a template pEA10
10 (Nakari-Setälä et al. 1996) and as a 5' primer GAC CTC GAT GCC CGC CCG GGG TCA AG (SEQ ID 33) and as a 3' primer GTC GAC ATT TCA TTT TAC CCC CCT CG (SEQ ID 34). The underlined sequences in the 5' and 3' primers are SacI and SalI sites, respectively. The PCR fragment was cut with SacI and SalI so that the SacI site was blunted with Klenow. The fragment was cloned into cSC3/pUC20 (described in Example
15 25) vector digested with SalI and BamHI where the BamHI was blunted with Klenow. In the next step, the *hfb2* promoter (SEQ ID 35) was amplified with PCR using as a template pTNS8 (Nakari-Setälä et al. 1997) and using as a 5' primer AAG CTT GCA TGC CTG CAT CC (SEQ ID 36) and as a 3' primer CCA TGG TGA AAG GTG GTG ATG GTT GG (SEQ ID 37). The underlined sequences in the 5' and 3' primers are HindIII and
20 NcoI sites, respectively. The PCR fragment was digested with HindIII and NcoI and cloned in front of the SC3 cDNA in the plasmid obtained in the previous step and cut with the same restriction enzymes. The resulting plasmid is pKS2 (Figure 23).

Example 29

25 **Construction of *T. reesei* strains producing EGIcore-HFBII, EGIcore-SC3, HFBI-dCBD and HFBI-single chain antibody fusion proteins, and SC3 hydrophobin**

Trichoderma reesei strains VTT-D-74075 (QM9414), VTT-D-86271 (Rut-C30) and VTT-D-99676 (Rut-C30 Δ*hfb2*) were co-transformed essentially as described (Penttilä *et al.*,
30 1987) using 3-13 µg of the plasmids pTNS32, pTH1, pTH2, pTH4, pTNS30 and pKS2 and as the selection plasmids 1-3 µg pToC202 or pARO21. pToC202 carrying the *amdS* gene (Hynes *et al.*, 1983; Tilburn *et al.*, 1983) of *A. nidulans* and pARO21 carrying the *E. coli hph*

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gene have been described in Example 3. The Amd+ and Hyg+ transformants obtained were streaked three times onto plates containing acetamide and hygromycin, respectively (Penttilä *et al.*, 1987). Thereafter spore suspensions were made from transformants grown on Potato Dextrose agar (Difco).

5

The production of the fusion proteins EGIcore-HFBII, EGIcore-SC3, HFBI-dCBD and HFBI-ENA5ScFv and the SC3 hydrophobin was tested by slot blotting or Western analysis with EGI, SC3, CBD and HFBI specific antibodies from shake flask or microtiter plate cultivations carried out in minimal medium supplemented with either lactose or Solka flock

10   cellulose. The spore suspensions of the fusion protein producing clones were purified to single spore cultures on selection plates (containing either acetamide or hygromycin). To determine the best producers, production of the fusion proteins was analyzed again from these purified clones as described above.

15   *T. reesei* strains selected for further cultivations are X46A (pTNS32, host QM9414), VTT-D-00793 (pTH4, host Rut-C30 Δhfb2), VTT-D-99727 (pTNS30, host Rut-C30 Δhfb2), VTT-D-00791 (pTH1, host Rut-C30) and VTT-D-00792 (pKS2, host Rut-C30 Δhfb2). These strains were cultivated as described in Example 4 and the culture supernatants were subjected to ATPS.

20

Example 30

Recovery of the protein product from the enriched detergent phase after ATPS by isobutanol or other solvents

25   After phase separation has occurred and the hydrophobin or the hydrophobin fusion protein is enriched in the detergent phase (the enriched phase), the protein can be recovered to an aqueous buffer by addition of isobutanol or other solvent. For example a series, of identical extractions of 0.05 g/l HFBI in 50 mM acetate buffer was made with 2% Berol 532. To separate tubes 10% final concentration of either isobutanol, n-amyl

30   alcohol, octanol, or octane was added. and the fraction of hydrophobin recovered to the aqueous phase was analyzed. It was found that 100% was recovered with isobutanol,

89% with n-amyl alcohol, 81 % with octanol and 70 % with octane. Fractions were analyzed by HPLC as described in Example 38.

5   **Example 31**

Separation of EGIcore-HFBII fusion protein in ATPS.

T.reesei strain X46A was grown in shake flasks on 3% lactose medium as described in Example 4. C12-18EO5 detergent was added to a final concentration of 5% to the culture supernatant. After mixing the system was allowed to settle and the enriched

10   detergent phase was subjected to further extraction with an equal volume of isobutanol. After buffer exchange on Biogel P-6 (Bio-Rad, USA) gel filtration media, the extracted protein was analysed by ion exchange chromatography using a Mono Q (Amersham Pharmacia, Sweden) column equilibriated with 15 mM acetate and eluted using a linear gradient of NaCl. Separation of the EGIcore-HFBII fusion protein to the detergent phase

15   was verified by analyzing the eluted peak fraction from the Mono Q chromatography run by its activity on 4-methylumbelliferyl cellobioside as well as its re-extraction by ATPS.

Example 32

Separation of EGIcore-SC3 fusion protein in ATPS

20

Culture filtrate of *T.reesei* strain VTT-D-00793 producing EGIcore-SC3 fusion protein was subjected to APTS by adding 5% final concentration of C12-18EO5. After the settling, the phases were separated and the fusion protein enriched detergent phase was further treated with an equal volume of isobutanol which removes the detergent to the

25   isobutanol and leaves the protein in the aqueous phase. The resulting fusion protein enriched aqueous phase was then desalted on Biogel P-6 (Biorad, USA). Separation of fusion protein to the detergent phase was verified by analyzing the enzymatic activity of the EGIcore fusion partner on 4-methylumbelliferyl cellobioside in that phase.

30

Example 33

Separation of HFBl-dCBD fusion protein in ATPS

T.reesei strain VTT-D-99727 producing HFBI-dCBD fusion protein was grown on
5   lactose-containing medium as described in Example 4. Berol 532 was added to a final
concentration of 2 % to 500 ml of culture supernatant. The mixture was allowed to settle
and the phases separated in a separation funnel. The enriched top phase (10 ml) was
extracted with an equal volume of isobutanol and 50 mM acetate buffer pH5. The fusion
protein was selectively enriched to the buffer in the separation as shown by SDS gel
10   electrophoresis (Figure 24).

Example 34

Recovery of the HFBI-dCBD fusion partners after chemical cleavage

15   HFBI-dCBD protein produced by the strain VTT-D-99727 has a methionine
(PGASTSTGMGPGG) designed in the linker region between the HFBI and the dCBD,
which would enable the recovery of the native HFBI and dCBD after chemical cleavage with
CNBr.

20   The HFBI-dCBD fusion protein was purified as described in Example 33. The resulting water
phase (~108 ml) still contained a small amount of CBHI and free hydrophobin in addition to
the HFBI-dCBD fusion (Figure 24). Further purification of the sample was performed by
chromatography. The sample was desalted on a Biogel P-6 column equilibrated with 50mM
sodium acetate buffer pH 5.5, diluted 1 + 3 with water and applied on a CM-Sepharose FF
25   column equilibrated with 10mM sodium acetate buffer pH 5.5. The CBHI protein was found
in the flow through and the purified HFBI-dCBD protein was eluted with 0.2M NaCl.

A sample of purified HFBI-dCBD protein was evaporated in speed-vac almost to dryness.
CNBr cleavage was performed by adding a solution of CNBr in 0.1M HCl (5mg/ml) to the
30   evaporated sample in 5 times weight excess. The reaction was allowed to continue for 24h at
room temperature (in dark). A 10 times volume of water was added and the sample was

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evaporated in speed-vac almost to dryness. The evaporated sample was diluted in 50mM Tris-HCl pH 7 and the cleavage was confirmed by HPLC analysis as described in Example 37.

5   The treatment was further optimised by using different concentrations of HCl and incubation times. The final CNBr treated sample was subjected to ATPS as described in Example 33. HFBI was found in the top-phase and the dCBD in the bottom phase, thus leading to a separation of both fusion partners.

10   **Example 35**

Separation of HFBI-single chain antibody fusion protein in ATPS

Strain VTT-D-00791 was cultivated for 7 days on medium containing 10 g/l potassium phtalate, 15 g/l KH_2PO_4, 5 g/L $(NH_4)_2SO_4$ and 3% lactose and 0.2% peptone. 30 ml of

15   culture medium was subjected to ATPS using 4% C12-18-EO5 detergent as described in Example 5. After removal of the bottom phase, the detergent phase was extracted by isobutanol. Proteins from the water phase after detergent extraction and the bottom phase of the isobutanol extracted detergent phase were precipitated with trichloroacetic acid (final concentration 10%), resuspended in SDS-PAGE sample buffer (taking into acount the

20   concentration factor of the isobutanol extracted detergent phase) and analysed by Western blotting using HFBI specific antibodies. The fusion protein runs as a dimer (approximately 70 kDa) in SDS-PAGE under the conditions used as seen in Figure 25. The water phase sample still contained a small amount of fusion protein. Nevertheless, separation of the fusion protein can be obtained by ATPS.

25

Example 36

Separation of SC3 hydrophobin in ATPS

SC3 hydrophobin producing strain VTT-D-00792 was cultivated in shake flasks on lactose-

30   containing medium as described in Example 4. Soluble secreted proteins produced by the fungus were precipitated with trichloroacetic acid. Precipitated proteins were solubilized in trifluoroacetic acid to dissociate the SC3 aggregates whereafter the acid was evaporated with

a stream of air. The treated proteins were solubilized in 2 ml water containing 2.5 % of the detergent C12-18EO5. Phase separation was carried out as described in Example 5. Samples were taken from the lighter and heavier phase and analysed by Western blotting using SC3 specific antibodies. On the basis of this analysis, SC3 hydrophobin partitions to the detergent

5   phase (Figure 26).

Example 37

Separation of nisin in ATPS

10   1 mg of pure nisin (Sigma) (equals to 1000 IU) in 50 mM sodium acetate buffer pH 5 was subjected to ATPS using 2% of the detergent C12-18EO5 at 30°C, and the phases were allowed to settle. After removal of the bottom phase, the detergent phase was extracted by isobutanol which removes the detergent to the isobutanol and leaves nisin in the aqueous phase. Separation of nisin to the enriched aqueous phase was assayd testing nisin

15   bioactivity as described by Qiao et al. 1996. Comparison of the halos on the assay plate *produced by the enriched phase and the nisin controls indicated that nisin had separated* to the detergent phase with a concentration factor of approximately 5.

20   **Example 38**

Preparative purification of HFBI and HFBII in ATPS

For preparative purposes, HFBI and HFBI were extracted from 0.5 l of culture supernantants from cultivations carried out on media containing glucose or cellulose as

25   described in Example 4. 2% (w/w) detergent was added to the culture supernatant which was mixed and then allowed to settle in a separation funnel at 20°C if C11EO2 (Berol 532) *was used and 30°C if C12-18EO5 (Henkel) was used.* The detergent phase (enriched phase) was collected and mixed with an equal volume of isobutanol. When C11EO2 was used an equal volume of 50 mM acetate buffer was also added. The

30   remaining culture supernatant is the depleted phase. To follow the purification, analytical HPLC was run on samples from each step (Figure 27and 28). On the basis of HPLC analysis, both HFBI and HFBII partitioned well to both of the detergents. When

C11EO2 was used, K values of >1000 and >78 were obtained for HFBI and HFBII, respectively.

Water phase after isobutanol extraction was collected and furher purified on a Vydac
5   1x20 cm semipreparative C4 column (Vydac, USA) eqilibriated with 0.1% trifluoro acetic acid in water and eluting with a linear gradient of 0.1% trifluoro acetic acid in acetonitrile. The proteins were then lyophilized. In analytical runs a 4.6 mmx20 cm Vydac C4 column was used.

10   **Example 39**

Screening for separation conditions using purified HFBI and HFBII

HFBI and HFBII which were purified from culture supernatants in ATPS, further purified by preparative HPLC and lyophilized as described in Example 38 were dissolved
15   in water to a concentration of 0.5 mg/ml. The stock solutions were then diluted with the buffer appropriate for the experiment. 50 mM acetate buffer pH5, 50 mM glycine pH 3, 50 mM HEPES pH 7 was used for pH screening and NaCl or $(NH_4)_2SO_4$ in acetate buffer was used for ionic strength experiments. The surfactant was weighed with the appropriate amount of hydrophobin solution and incubated in 0, 20, 30, 40°C and the
20   amount of hydrophobin in the depleted phase determined. Partitioning coefficients were calculated from the change in concentration and the volume ratios and are presented in the tables below.

25

30

Table presenting K-values for the separation of hydrophobins with different surfactants and temperature.

	Temperature	Henkel	Berol 532	Berol 266	Triton x114
HFBI	0°C	0,1	368	NP	NP
	20°C	0,4	1961	NP	NP
	30°C	85	2970	NP	0,9
	40°C	148	682	28	69
HFBII	0°C	NP	76	NP	NP
	20°C	1,0	139	NP	NP
	30°C	67	194	NP	4,0
	40°C	44	102	19	44

5   NP = no phase separation

Table presenting the effect of different salt concentrations at 40°C on K-values
10

		Henkel	Berol 532	Berol 266	Triton x114
HFBI	50 mM buffer	244	578	13	39
	+ 1 M NaCl	239	33	198	241
	+1 M $(NH_4)_2SO_4$	0,9	3,2	176	612
HFBII	50 mM buffer	36	80	16	58
	+ 1 M NaCl	14	87	61	38
	+1 M $(NH_4)_2SO_4$	3,1	351	95	35

Table presenting the K-values for the partitioning of hydrophobins in Berol 532 at
15 different conditions.

	HFBI 0°C	HFBI 20°C	HFBII 0°C	HFBII 20°C
50 mM acetate pH5	298	614	76	127
+1 M NaCl	614	3781	131	199
+1M $(NH_4)_2SO_4$	931	44	203	139
pH3	614	1881	73	160
pH7	361	614	54	126

Example 40

Purification of a new putative *T. reesei* hydrophobin on the basis of its separation in ATPS, and cloning of the corresponding gene

5   *T. reesei* strain VTT-D-99726 (QM9414 Δ*hfb2*) was cultivated on lactose-based medium in 15 fermentor as described in Example 4. After cultivation, 1 l of the culture filtrate was subjected to ATPS with 5% polyoxyethylene detergent C12-18EO5. After phase separation at 30°C, detergent phase was separated from the depleted aqueous phase and analysed by SDS-PAGE. The analysed sample contained a protein which

10   was about 7.5 kDa. In addition, some larger proteins were present. These were removed by hydrophobic interaction chromatography on a Phenyl-Sepharose FF column equilibriated with 50 mM sodium acetate - 1 M (NH$_4$)$_2$SO$_4$ buffer. The proteins were eluted with descending (NH$_4$)$_2$SO$_4$ salt gradient. Fractions containing the ca. 7.5 kDa protein were pooled and concentrated, and analysed by mass

15   spectrometry. This revealed that the purified protein was degraded to three peptides (2486, 2586 and 2709 Da), which were held together by disulphide linkages. The N-terminal amino acid sequence from the 2486 Da peptide was ANAFCPEGLLYTNPLCCDLL, which on the basis of position of cysteines and on sequence comparison to known hydrophobins is typical to a hydrophobin.

20

Degenerated primers were designed on the basis of this amino acid sequence as well as on the basis of the sequence obtained from the 2586 Da peptide. These primers were used in RT-PCR (RobusT RT-PCR Kit, Finnzymes) with RNA isolated from the same cultivation from where the protein was purified. The 140 bp fragment obtained

25   from the RT-PCR reaction was sequenced. The obtained sequence contained a part that was coding for the 2486 Da peptide used to design the PCR primer confirming that the PCR product corresponds to the purified protein.

30

Example 41

Purification of EGIcore-HFBI in ATPS in pilot scale

The strain VTT-D-99702 producing EGIcore-HFBI fusion under the *cbh1* promoter was
5   cultivated in pilot scale fermentor in 1200 L on minimal medium containing 4% lactose,
0.4 % peptone and 0.1% Yeast Extract for 4 days. Cultivation temperatures were 28°C
or alternatively the temparature was changed during the cultivation from 27°C to 22°C in
a step-wise manner. Production level of the fusion protein was a few grams per liter.
After the end of the cultivation, the mycelium was separated by means of a rotating
10 vacuum drum filter using Celite 535 diatomaceous earth as filter aid.

In separation 1, 1100 L of the supernatant was transferred into the meanwhile cleaned
bioreactor, adjusted to the separation temperature of 24,7 °C and mixed with salt and
detergent to give 0,15 M ammonium dihydrogen phosphate and 4,1 % of detergent C12-
15 18EO5. The phases were left to separate by gravity settling and the heavier phase
removed through the bottom valve. A parallel experiment in 10 ml volume was carried
out to investigate the effect of scale up on the separation of the fusion protein. The
partition coefficient and the concentration factor were equal for the 10 ml and 1200 L
separation within the measurement error. The separated detergent phase was re-
20 extracted using the detergent phase from the first extraction by replacing half of the
volume of the bottom phase by tap water. The salt concentration was adjusted to 0,25 M
ammonium dihydrogen phosphate at 30°C. K and Y values of the separations are
presented in the below Table.

25 Alternatively in separation 2, 10 ml of drum filtrated culture medium samples were
subjected to ATPS at 25°C using 4.1% C12-18EO5 detergent and 1.15 M $(NH_4)H_2PO_4$.
K and Y values after one separation step are presented in the Table below.

30

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Table presenting extraction experiments of culture supernatants from different pilot scale
cultivations

Separation	Cultivation temperature	Concentration factor	K	Y [%]
Separation 1 Re-extraction	27°C → 22°C	3.4	3.8 8	59 70
Separation 2	29°C	2.8	8.3	81

Example 42

**Separation of EGI, EGIcore-HFBI fusion protein and HFBI hydrophobin in robust
micelle/polymer systems**

Separations were performed with purified samples. HFBl was purified by two-phase
separation and HPLC as decribed in Example 38. EGIcore-HFBI was purified by two-phase
extraction as decribed in Example 5 followed by desalting on a Biogel P-6 column
equiliberated with 20mM sodium acetate puffer pH 6 containing 150mM NaCl. The purified
proteins were subjected to ATPS using different micelle/polymer systems.

The table below presents partitioning coefficients of pure EGI, HFBI and EGIcore-HFBI
at constant tie-line length, in the different systems, Triton X-114/water system (4.1 %
wt.) at 31.5° C, Triton X-100/Reppal/water system (8.1 % wt. / 8.2 % wt.) and Triton
X-114/Reppal/water system (5.0 % wt. / 4.0 % wt.) at 21° C. The standard deviation is
shown within the brackets. The systems were buffered with 25 mM sodium acetate, pH
4.0. K > 1 is equivalent to a preferred protein partitioning to the micelle rich phase.

	EGI	HFBI	EGIcore-HFBI	
	K_{EGI}	K_{HFB}	K_{EGIHFB}	Yield (%) top phase
Triton X-114	0.6 (0.1)	21.5 (1.5)	8.4 (1.0)	85
Triton X-100/Reppal	0.9 (0.1)	-	1.7 (0.1)	56
Triton X-114/Reppal	0.7 (0.1)	11.0 (1.1)	15.4 (2.9)	91

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